

Relationship of the 37,000- and 40,000-*M_r* Tryptic Fragments of Islet Antigens in Insulin-dependent Diabetes to the Protein Tyrosine Phosphatase-like Molecule IA-2 (ICA512)

Mark A. Payton, Charlotte J. Hawkes, and Michael R. Christie

Department of Medicine, King's College School of Medicine and Dentistry, London SE5 9PJ, United Kingdom

Abstract

Sera from patients with insulin-dependent diabetes immunoprecipitate 64,000-*M_r* proteins, distinct from glutamate decarboxylase, that are cleaved to 37,000- and 40,000-*M_r* fragments by trypsin. We investigated possible relationships between 37,000- or 40,000-*M_r* fragments of antigen and the tyrosine phosphatase-like protein, IA-2 (ICA512). Antibodies from nondiabetic relatives bound differentially to 37,000- and 40,000-*M_r* fragments indicating presence of distinct epitopes. Precursors of these fragments could be separated on immobilized lectins, suggesting different carbohydrate content. Levels of antibodies to 40,000-*M_r* fragments were strongly associated with those to the intracellular domain of IA-2. Recombinant intracellular domain of IA-2 blocked binding of antibodies to 40,000-*M_r* fragments expressed by insulinoma cells and partially blocked binding to 37,000-*M_r* fragments. Furthermore, trypsinization of recombinant intracellular domain of IA-2 generated proteolytic fragments of identical *M_r* to the 40,000-*M_r* fragments of insulinoma antigen; 37,000-*M_r* fragments were not generated. Thus, 40,000-*M_r* fragments of islet autoantigen are derived from a protein similar or identical to the tyrosine phosphatase-like molecule, IA-2. The 37,000-*M_r* fragments are derived from a different, although related, protein. (*J. Clin. Invest.* 1995. 1506–1511.) **Key words:** insulin-dependent diabetes • autoantigens • autoantibodies • protein tyrosine phosphatase • glycoproteins

Introduction

Patients with insulin-dependent diabetes mellitus (IDDM)¹ possess antibodies to multiple islet cell antigens including insulin, the 65-kD isoform of glutamate decarboxylase (GAD), and carboxypeptidase H (1). In our laboratory we have been characterizing protein autoantigens in IDDM that are detected on SDS-

PAGE as diffuse 64,000-*M_r* bands but cleaved by trypsin to 37,000- and 40,000-*M_r* proteolytic fragments (37k and 40k antigens) (2). Antibodies to these antigens are found in up to 80% of recent onset IDDM patients and are very closely associated with diabetes development in identical twins (3) and first-degree relatives of IDDM patients (4), in patients with polyendocrine autoimmunity or stiff-man syndrome (5), and in islet cell antibody (ICA)-positive schoolchildren (6). These antibodies should be particularly useful in identifying individuals at risk for IDDM who might be candidates for disease prevention protocols.

Identification of the 37k and 40k antigens is essential for the development of better assays for antibodies to the proteins for screening purposes and to assess the role of the antigens in disease pathogenesis. The antigens are expressed at low levels in pancreatic islets and insulinoma cells, and our attempts to purify sufficient quantities of protein for amino acid sequencing or antibody production have not been successful. As an alternative approach, we have compared the physical and antigenic properties of the proteins with potential autoantigens identified in other studies. We now report that the 37,000- and 40,000-*M_r* fragments are derived from different, but probably related, protein precursors. Furthermore, we demonstrate that the 40,000-*M_r* fragments are related to a protein designated IA-2 (7), which is homologous to members of the receptor-linked family of protein tyrosine phosphatases and which shares sequence identity with a cloned target for antibodies in human IDDM, ICA512 (8).

Methods

Sera. Sera from recent onset IDDM patients, from first-degree relatives of IDDM patients, and from healthy controls, previously characterized for antibodies to islet 64,000-*M_r* proteins, to GAD, and to tryptic fragments of islet antigen were selected from previous studies on autoantigens in IDDM (3, 9–11).

Islets and cell lines. Islets were isolated from 5–7-day-old newborn Wistar rats as described (12). The rat insulinoma cell line subclone RIN-5AH (13) was maintained in tissue culture in RPMI 1640 medium (GIBCO BRL, Paisley, UK) containing 10% FCS (Sigma Chemical Co., Poole, Dorset, UK). Endogenous proteins in islets and insulinoma cells were labeled with [³⁵S]methionine (Amersham International, Little Chalfont, UK) as described (9).

cDNA cloning and expression. RNA was extracted from human fetal brain (gift of Dr. Kate Langford, King's College School of Medicine and Dentistry, London) using Tri reagent™ (Molecular Research Center, Inc., Cincinnati, OH). First-strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) using poly-A and random primers. Human islet cDNA was a gift of Dr. Paul Banga (King's College School of Medicine and Dentistry, London). A segment of ICA512 DNA sequence was amplified from human fetal brain cDNA with Taq DNA polymerase using primer sequences: 5'-ATGAACCGG-GCAGAGGGTCCA-3' and 5'-TCATCAATCTCCTTCACTCCT-3'. Amplification products were reamplified with the same primer sequences but with additional sequences added at 5' ends corresponding to restric-

Address correspondence to Dr. M. R. Christie, Department of Medicine, King's College School of Medicine and Dentistry, Bessemer Road, London SE5 9PJ, United Kingdom. Phone: 44-171-737-4000, Ext. 4216; FAX: 44-171-346-3313.

Received for publication 17 February 1995 and accepted in revised form 2 June 1995.

1. **Abbreviations used in this paper:** 37k and 40k antigens, 37,000- and 40,000-*M_r* proteolytic fragments; GAD, glutamate decarboxylase; IA-2ic, intracellular domain of IA-2; ICA, islet cell antibody; IDDM, insulin-dependent (type 1) diabetes mellitus.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/95/09/1506/06 \$2.00

Volume 96, September 1995, 1506–1511

tion sites for BamHI and EcoRI, respectively. Reamplification products were ligated into the BamHI and EcoRI sites of the vector pGEM 4Z (Promega Corp., Madison, WI). DNA sequence corresponding to the intracellular domain of IA-2 (IA-2ic) was amplified from human islet cDNA using the primer sequences: 5'-ATGCAGCAAGACAAGGAGCGCTG-3' and 5'-TCACTGGGGCAGGGCCTTGAG-3'. Amplification products were cloned directly into the vector pGEM-T (Promega) and subcloned into pGEM 4Z and PinPoint™ (Promega).

A 3.5-kb cDNA containing the coding region for the rat homologue of the IA-2 molecule was obtained by screening a rat brain cDNA library in λ -ZAP (Stratagene Ltd., Cambridge, UK) with the human ICA512 DNA segment. The cDNA was subcloned into pGEM 11Z (Promega). A 5.3-kb cDNA for the 65-kD isoform of rat GAD was also cloned from the rat brain library by screening with a 346-bp rat GAD₆₅ DNA segment generated by DNA amplification. The coding region for rat GAD₆₅ was amplified from the 5.3-kb cDNA using specific primers and cloned into pGEM 4Z.

Nucleotide sequences of cloned products were verified by automated sequencing using a sequencer (ABI 373A; Applied Biosystems Inc., Foster City, CA). ICA512, IA-2ic, and GAD sequences in pGEM 4Z or pGEM 11Z were transcribed and translated in vitro in the presence of [³⁵S]methionine (Amersham) using the TNT™-coupled reticulocyte lysate system (Promega). IA-2ic was also expressed in *Escherichia coli* as a fusion protein with a biotin-labeled peptide sequence at the NH₂ terminus using the PinPoint™ expression system. Bacteria were lysed by stirring with lysozyme (1 mg/ml) in 150 mM NaCl, 10 mM Hepes, pH 7.4, followed by addition of Triton X-100 (0.1% vol/vol) for 5 min and DNase (20 U/ml) for 5 min. Particulate material was sedimented by centrifugation at 10,000 g for 15 min. The expressed fusion protein was purified from lysates by affinity chromatography on streptavidin-polymethacrylate (Soft Link™, Promega) columns. Protein was eluted with 5 mM biotin, which was subsequently removed by dialysis against 1 mM sodium phosphate, pH 7.4. The purified IA-2ic fusion protein resolved on SDS-PAGE as a single Coomassie brilliant blue-stained band with an *M_r* of 59,000.

Immunoprecipitation. Radiolabeled islets or insulinoma cells were extracted in Triton X-114 and subject to detergent-phase separation as previously described (2). Rat IA-2, human IA-2ic, or rat GAD₆₅ cDNA in pGEM-4Z, and cDNA for human GAD₆₅ in the vector pB1882 (gift of Dr. Thomas Dyrberg, Novo Nordisk, Copenhagen, Denmark) were transcribed and translated in vitro using commercial kits (Promega) in the presence of [³⁵S]methionine (Amersham). Incorporated radioactivity was determined by precipitation with 10% TCA and scintillation counting. Aliquots (20 μ l) containing 5×10^6 cpm of radiolabeled protein in cell extracts or 20,000 cpm of in vitro translated protein were incubated in immunoprecipitation buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.5 mg/ml BSA, 10 mM benzamidine, and 5 mM methionine) with 5 μ l of test serum for 5 h at 4°C. In some experiments, purified IA-2ic fusion protein was added before addition of serum. Immune complexes were isolated on 5 μ l of protein A-Sepharose and immunoprecipitates washed five times with 500 μ l of immunoprecipitation buffer and once with 500 μ l of water. Immune complexes were analyzed by SDS-PAGE and autoradiography. In some experiments with in vitro translated proteins, immunoprecipitates were resuspended in 500 μ l of water, and radioactivity in 200- μ l aliquots was determined by liquid scintillation counting. Radioactivity in immunoprecipitates was expressed as a percentage of that precipitated by a positive control serum included in each experiment.

Lectin affinity chromatography. Triton X-114 detergent phase-purified extracts of [³⁵S]methionine-labeled RIN-5AH cells were incubated with 50 μ l of the lectins immobilized on Sepharose or agarose at 4°C for 30 min and subsequently washed three times with immunoprecipitation buffer. Bound proteins were eluted three times with 50 μ l of 0.5 M α -methyl mannoside (lentil lectin, concanavalin A), 0.5 M *N*-acetyl glucosamine (wheat germ agglutinin), 0.5 M *N*-acetyl galactosamine (soybean agglutinin), or 0.5 M *N*-acetyl neuraminic acid (slug agglutinin). Eluates were subjected to immunoprecipitation with sera positive and negative for antibodies to 37k antigen as described above.

Database searches. Searches of the GenBank DNA sequence data-

base and DNA sequence analyses were performed with the University of Wisconsin Genetics Computer Group programs (14).

Statistical analysis. The degree of correlation among antibody measurements on serum samples was determined by linear regression analysis.

Results

Binding of antibodies from relatives of IDDM patients to 37,000- and 40,000-*M_r* fragments of antigen. We have previously reported a strong correlation between the presence of antibodies to 37,000- and 40,000-*M_r* fragments of antigen in sera from IDDM patients (15). These results suggested that the proteolytic fragments have similar antigenic properties and may be derived from the same or related proteins. However, analysis of binding of antibodies in sera of nondiabetic relatives of IDDM patients has revealed differences in antibody reactivity to the two fragments. Thus, in addition to sera that contain antibodies to both 37,000- and 40,000-*M_r* fragments, sera were identified that contain antibodies only to the 40,000-*M_r* fragments (Fig. 1 A). These results suggest that there may be differences in the antigenic properties of these antigenic fragments and that they may not necessarily be derived from the same protein.

Binding of precursors to the 37,000- and 40,000-*M_r* fragments to lectins. Further evidence that the 37,000- and 40,000-*M_r* fragments are not derived from the same protein was obtained by analysis of binding of antigen to immobilized lectins. Solubilized extracts of [³⁵S]methionine-labeled RIN-5AH insulinoma cells were applied to different immobilized lectins, bound proteins eluted with appropriate sugars, and eluates analyzed for the presence of autoantigens by immunoprecipitation and trypsin treatment. Precursors to 40,000-*M_r* fragments were recovered in eluates of wheat germ agglutinin columns (specificity *N*-acetyl glucosamine) whereas those of 37,000-*M_r* fragments were absent (Table I). Precursors of 37,000-*M_r* fragments were recovered in eluates of lentil lectin columns (see Table I, mannose specificity). These results demonstrate that these autoantigens are glycoproteins and that the precursors to the 37,000- and 40,000-*M_r* fragments have different carbohydrate content.

Correlation of antibodies to 40,000-*M_r* fragments with antibodies to ICA512 and IA-2. Screening of islet cDNA libraries with sera from IDDM patients has recovered cDNA for a number of potential IDDM-associated autoantigens, including a clone designated ICA512, that contains a domain homologous to protein tyrosine phosphatases (8). Analysis of the predicted amino acid sequence of this protein revealed a number of similarities with the 37k and 40k antigens, including a predicted molecular mass of ~ 60 kD, the presence of potential *N*-glycosylation sites, and a putative transmembrane domain, consistent with the known amphiphilic properties of the 37k- and 40k-antigens (2). A segment of cDNA containing the reported antibody binding site on ICA512 (Fig. 2) was obtained by DNA amplification from human fetal brain cDNA and cloned into the vector pGEM 4Z. Protein labeled with [³⁵S]methionine was generated by in vitro transcription and translation and immunoprecipitated with sera of known reactivity with 37,000- and 40,000-*M_r* antigenic fragments. Sera from IDDM patients and relatives, but not healthy controls, were found to immunoprecipitate the in vitro translated protein (Fig. 1 B). All sera that immunoprecipitated the protein were found to be positive for antibodies to 40,000-*M_r* fragments of islet autoantigen. How-

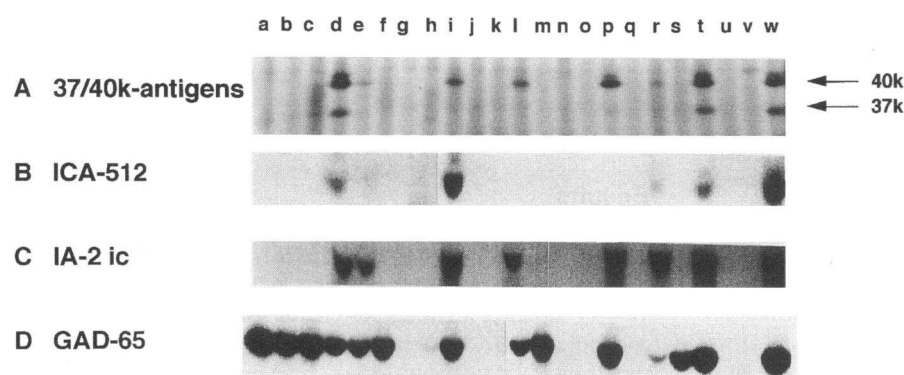


Figure 1. Immunoprecipitation of insulinoma cell or recombinant antigens by antibodies in sera of relatives of IDDM patients. (A) Sera from 23 nondiabetic, ICA-positive relatives (a–w) of IDDM patients were used to immunoprecipitate proteins from Triton X-114 extracts of [35 S] methionine-labeled RIN cells. Immune complexes were isolated on protein A–Sepharose and treated with trypsin (0.1 mg/ml) before SDS-PAGE and autoradiography. Antigens were detected as 40,000- M_r fragments (upper bands in A, lanes d, e, i, l, p, r, t, and w) or 37,000- M_r bands (lower bands in A, lanes d, t, and w).

The same sera were used to immunoprecipitate in vitro transcribed and translated proteins representing the reported ICA512-1 sequence (B), IA-2ic (C), or human GAD-65 (D). Immunoprecipitated proteins were resolved on SDS-PAGE and visualized by autoradiography.

ever, not all sera positive for the antibodies to the islet antigen immunoprecipitated the translated product (compare Fig. 1, A and B). The ICA512 segment cloned by us is therefore not fully homologous to the islet 37k or 40k antigens.

A search of the GenBank database identified an additional cDNA sequence, IA-2/PTP, containing the complete ICA512 sequence but with extended sequence at the 5' end and an additional guanosine nucleotide within the coding region. The consequent frame-shift resulted in a longer than predicted protein sequence COOH-terminal to the tyrosine phosphatase domain, and the entire IA-2 sequence encodes a protein of a predicted molecular mass of 106 kD (7). A segment of human IA-2 cDNA representing the coding region 3' to the putative transmembrane domain (IA-2ic) was obtained by DNA amplification of human islet cDNA and cloned into pGEM 4Z. A cDNA clone including the coding region for the rat homologue of the complete IA-2 molecule was obtained from a rat brain library, and the cDNA was subcloned into pGEM 4Z. The corresponding [35 S]methionine-labeled proteins were transcribed and translated in vitro. The dominant translation products were of appropriate size (42,000 and 105,000 M_r for IA-2ic and the complete rat IA-2 molecule, respectively) but minor products of smaller sizes were also detected which are probably the results of translation from intramolecular methionine codons. Reactivity with antibodies in sera from IDDM patients and relatives was determined. Virtually all sera positive for antibodies to 40,000- M_r fragments recognized the IA-2ic product, in-

cluding sera negative for antibodies to the 37,000- M_r fragment (Fig. 1 C). Similar results were obtained for the complete rat IA-2 molecule. Furthermore, a strong correlation between levels of antibodies to IA-2ic and the 40,000- M_r fragments was observed ($r = 0.85$; $P \leq 0.001$; Fig. 3). No association between antibodies to IA-2ic and antibodies to GAD was detected (Fig. 1 D). These results demonstrate that the 40,000- M_r fragments share similar antigenic properties with the putative cytoplasmic domain of IA-2.

Sera containing antibodies to IA-2ic immunoprecipitate 64,000- M_r protein from islet extracts. We have previously shown that the 37,000- and 40,000- M_r fragments are derived from proteins that are resolved as diffuse bands of M_r 64,000 on SDS-PAGE and that are distinct from GAD (2). We therefore investigated whether the presence of antibodies to IA-2ic in recent onset diabetic patients' sera was associated with the ability to immunoprecipitate 64,000- M_r proteins from islet extracts. Sera were also tested for the presence of antibodies to GAD. Antibodies in sera from IDDM patients immunoprecipitated 64,000- M_r proteins that were resolved as both sharp and diffuse bands on SDS-PAGE (Fig. 4 A); diffuse 64,000- M_r bands were detected in patients with high levels of antibodies to IA-2ic (Fig. 4 A, lanes 1, 5, 7, and 9) whereas sharp 64,000- M_r bands were associated with the presence of high levels of GAD antibodies (e.g., Fig. 4 A, lanes 4, 11, and 13). No protein bands of different M_r were consistently and specifically detected in immunoprecipitates with sera positive for these antibodies.

Blocking of binding of antibodies to 37,000- and 40,000- M_r fragments of autoantigen by IA-2ic. To further investigate the relationship of IA-2 to 37k- and 40k-antigens, the IA-2ic

Table 1. Binding of 37,000- and 40,000- M_r Fragments of Antigen to Lectins

Lectin	Specificity	37k fragments	40k fragments
Lentil lectin	Mannose	+	–
Concanavalin A	Mannose	–	–
Wheat germ agglutinin	N-acetyl glucosamine	–	++
Soybean agglutinin	N-acetyl galactosamine	–	–
Slug agglutinin	N-acetyl neuraminic acid	–	–

Lectins immobilized on Sepharose or agarose were incubated with detergent extracts of RIN-5AH insulinoma cells for 30 min, washed, and bound glycoproteins eluted with appropriate sugars. The presence of precursors to 37,000- (37k) and 40,000- M_r (40k) fragments was determined by immunoprecipitation with a strongly antibody-positive serum followed by analysis of trypsinized immune complexes by SDS-PAGE and autoradiography. Bands immunoprecipitated were scored as positive (+) or strongly positive (++).

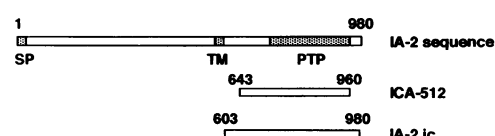


Figure 2. Location of cloned cDNA sequences within IA-2 sequence. Schematic representation of IA-2 sequence is shown. The shaded regions indicate locations of a putative signal peptide (SP), transmembrane domain (TM), and protein tyrosine phosphatase homology region (PTP). Two sequences were isolated by DNA amplification, one representing the ICA512 sequence containing autoantibody binding sites (residues 643–960) the other representing the entire intracellular domain of the protein (residues 603–980). cDNA for the complete IA-2 molecule was recovered from a rat brain cDNA library.

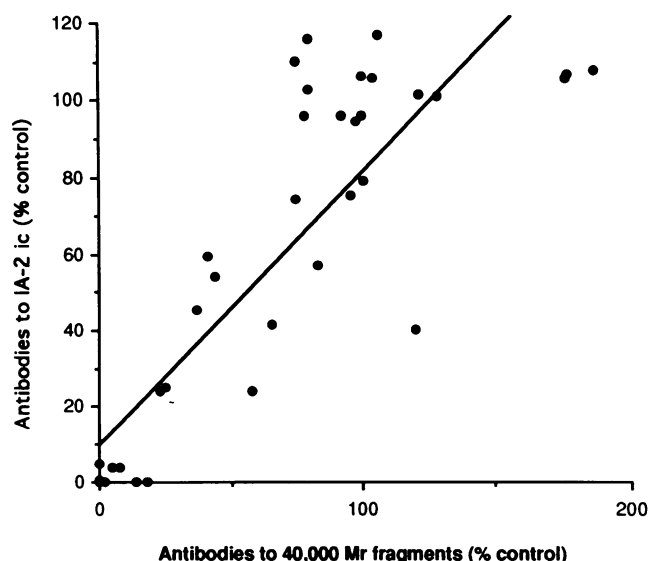


Figure 3. Association of antibodies to IA-2ic with antibodies to 40,000- M_r fragments of islet autoantigen. Levels of antibodies to 40,000- M_r fragments of islet antigen determined in previous studies were compared with levels of antibodies to IA-2ic in sera from 28 recent onset IDDM patients and nine first-degree relatives of IDDM patients. A strong association ($r = 0.85$; $P \leq 0.001$) was detected between these two antibody specificities.

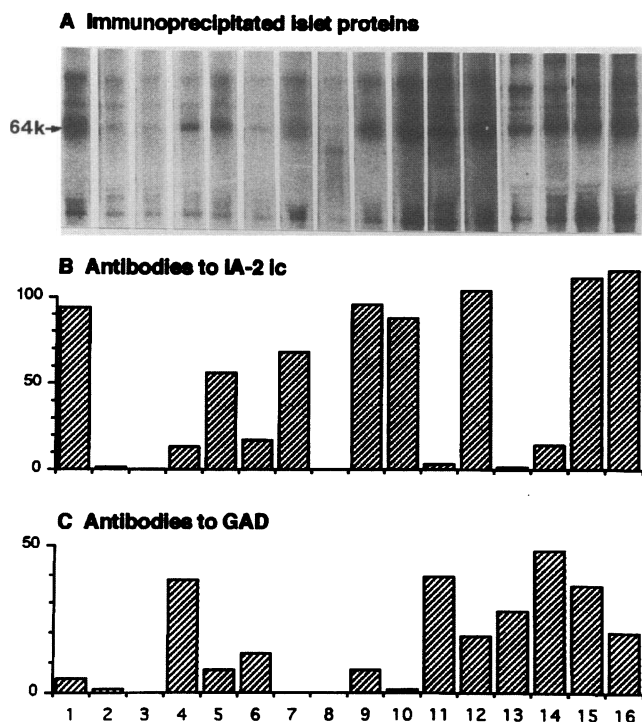


Figure 4. Antibodies to antigens in IDDM patients. (A) Autoradiogram showing proteins immunoprecipitated from Triton X-114 extracts of [35 S]methionine-labeled rat islets by sera from 16 recent onset IDDM patients. The arrow marks the position of the 64,000- M_r bands. Recombinant IA-2ic (B) and recombinant human GAD-65 (C), both expressed by in vitro transcription and translation, were immunoprecipitated by the same sera. Radiolabeled protein immunoprecipitated was determined by scintillation counting and expressed as a percentage of that precipitated by a positive control serum.

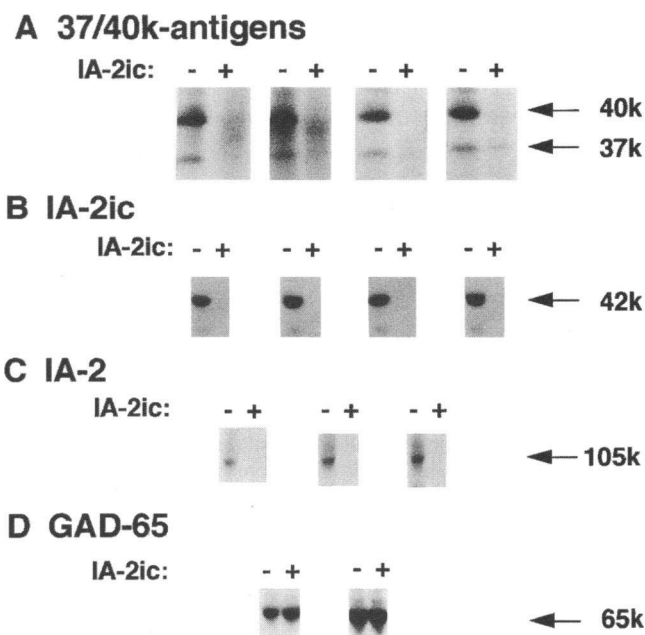


Figure 5. Blocking of binding of antibodies to antigens by IA-2ic. (A) Triton X-114 detergent extracts of RIN insulinoma cells were incubated with serum from four IDDM patients positive for antibodies to islet 37k-antigen with buffer only (left lane for each serum) or with buffer containing purified IA-2ic fusion protein (right lane for each serum). Autoradiograms showing proteins immunoprecipitated are illustrated. Sera from IDDM patients were also analyzed for ability of IA-2ic to block binding to human IA-2ic (B), rat IA-2 protein (C), and rat GAD $_{65}$ (D) expressed by in vitro transcription and translation. The M_r 's of the major radiolabeled proteins immunoprecipitated are marked.

cDNA was subcloned into the PinPointTM expression vector and expressed as a biotin-labeled fusion protein in *E. coli*. Expression of recombinant protein of appropriate size and antigenicity was confirmed by immunoprecipitation with sera from IDDM patients, followed by Western blotting and detection with alkaline phosphatase-conjugated streptavidin (data not shown). The ability of unlabeled recombinant IA-2ic to block binding to [35 S]methionine-labeled 37,000- and 40,000- M_r fragments of RIN cell antigen was determined. Addition of purified IA-2ic fusion protein blocked binding to both 37,000- and 40,000- M_r fragments of antigen, although with some sera blocking of binding to 37,000- M_r fragments was incomplete (Fig. 5 A). The IA-2ic preparation also blocked autoantibody binding to human IA-2ic (Fig. 5 B) and to the complete rat IA-2 molecule (Fig. 5 C) expressed by in vitro transcription and translation but had no effect on binding to GAD $_{65}$ (Fig. 5 D). These results further confirm the similar antigenic properties of IA-2 and the 40k-antigen.

Trypsinization of IA-2 generates 40,000- M_r fragments. Experiments were performed to determine whether trypsin treatment of IA-2 generates proteolytic fragments of similar size to those of native 37k- or 40k-antigen. Radiolabeled human IA-2ic and the complete rat IA-2 molecule were obtained by in vitro transcription and translation. Translated proteins were incubated with sera from IDDM patients and immunoprecipitated proteins either run directly on SDS-PAGE or treated with trypsin (0.1 mg/ml) for 20 min on ice before gel electrophoresis. The mobility on SDS-PAGE of the proteolyzed immunoprecipitates was compared with that of similarly treated immunoprecipitates with

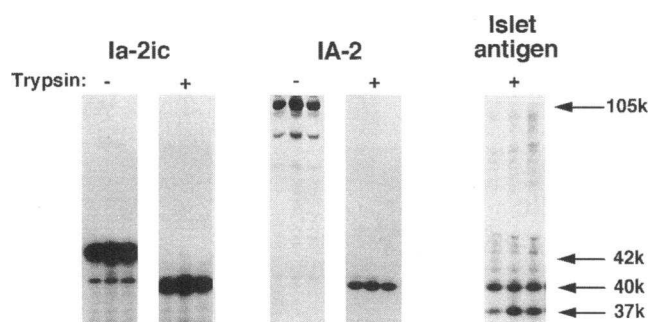


Figure 6. Trypsinization of immunoprecipitated IA-2 and islet antigen. IA-2ic (left panel) and the complete rat IA-2 protein (center panel) were expressed by transcription and translation in vitro and subjected to immunoprecipitation with IDDM patients' sera. Immunoprecipitates were analyzed directly by SDS-PAGE for proteins precipitated or treated with trypsin (0.1 mg/ml) for 20 min on ice before SDS-PAGE as indicated on the figure. The mobilities of bands detected were compared with those seen in trypsinized immunoprecipitates performed with Triton X-114 extracts of [35 S] methionine-labeled rat islets (right panel). The M_r 's of the major protein bands detected are marked.

RIN cells as source of 37k-antigen. Trypsin cleavage of both IA-2ic and the complete IA-2 protein generated a major fragment with identical mobility to the 40,000- M_r fragment of insulinoma antigen (Fig. 6). Fragments of 37,000 M_r were not generated under these conditions.

Discussion

A protein containing a domain homologous to the receptor-linked family of protein tyrosine phosphatases was first shown to be recognized by antibodies in IDDM by Rabin et al. (8). A clone (ICA512-1) encoding a portion (predicted molecular mass 33 kD) of the protein encompassing the tyrosine phosphatase domain was recovered from a human islet cDNA library by screening with antibodies from IDDM patients (16). The expressed protein was subsequently shown to be recognized by antibodies in sera of 48% of newly diagnosed IDDM patients by ELISA. Rescreening of the cDNA library identified a longer clone (ICA512-3) encoding a 60-kD protein, including a putative transmembrane domain and two potential *N*-glycosylation sites (8). Lan and colleagues (7) independently isolated a cDNA (IA-2) containing sequence virtually identical to ICA512 but extended at the 5' end, with a 3-kb open reading frame encoding a protein of predicted molecular mass of 106 kD. Neither group was able to demonstrate tyrosine phosphatase enzyme activity of the expressed protein using a variety of substrates. Furthermore, amino acid substitutions are present in IA-2 at highly conserved residues in other tyrosine phosphatases that have been shown to abolish enzyme activity towards synthetic substrates (17). Whether IA-2 (ICA512) has a very restricted substrate specificity or whether it is inactive as a tyrosine phosphatase remains to be determined.

In our initial studies on antibody binding to IA-2 and ICA512 we used protein expressed by a cloned cDNA encoding the reported protein sequence of ICA512-1, a region of the molecule known to contain the antibody binding domain (8). However, owing to a probable error in the ICA512 sequence, this protein has a COOH terminus truncated by 20 amino acid residues. We subsequently cloned the entire intracellular domain of IA-2, encoding a protein fragment (IA-2ic) 60 amino

acids longer than our ICA512 product. Virtually all sera positive for antibodies to 40,000- M_r fragments bound to IA-2ic, and a strong correlation between antibody levels was detected. Approximately 50% of sera positive for antibodies to IA-2ic also bound the shorter ICA512 polypeptide, showing that this region contains epitopes for some, but not all, of these diabetes-associated antibodies and revealing heterogeneity between patients in the epitopes recognized.

The strong correlation between antibodies to IA-2ic and the 40,000- M_r fragments indicate that these are antigenically similar. Other observations provide further evidence that these proteins are related. Thus, unlabeled IA-2ic blocked binding of antibodies to the 40,000- M_r antigenic fragment, and trypsinization of immunoprecipitated IA-2 and the cytoplasmic domain generated a polypeptide of identical M_r to these fragments. Sera containing high levels of antibodies to IA-2ic were shown to immunoprecipitate a protein migrating as a diffuse 64,000- M_r band on SDS-PAGE, as previously observed for precursors to the 40,000- M_r fragments (2). The predicted protein sequence has a putative transmembrane domain and potential *N*-glycosylation site; this is consistent with the known properties of the 37k- and 40k-antigens (2, 7). Together, these data suggest that the 40,000- M_r fragments are derived from a protein similar or identical to IA-2. Sera containing antibodies to 40k-antigen were able to immunoprecipitate the complete 105,000- M_r rat brain IA-2 protein expressed by in vitro transcription and translation. However, addition of excess cytoplasmic domain of the protein completely blocked autoantibody binding to the full-length molecule, suggesting that autoantibody recognition is restricted to the intracellular domain of the enzyme.

The predicted molecular mass of IA-2 (106 kD, including a putative signal peptide) is higher than that expected of the precursor to the 40,000- M_r fragments, which migrates on SDS-PAGE with an M_r of \sim 64,000 (Fig. 4 and reference 2). Only 64,000- and 38,000- M_r proteins have been detected by immunoprecipitation from non-protease-treated extracts of islets with diabetes-associated antibodies (18, 19). The M_r of native IA-2 has not been reported, so comparison of molecular weights must await further characterization of the natural protein. However, there are a number of possible reasons for the discrepancy. It is possible that the diffuse 64,000- M_r bands detected in immunoprecipitates with sera containing antibodies to 40k antigen are themselves degradation products of a higher molecular weight protein, although precautions were taken to inhibit proteolytic cleavage during cell extraction and immunoprecipitation. IA-2 molecule may be subject to proteolytic processing in vivo, or posttranslational modifications, such as glycosylation, may yield a protein that migrates at a lower than expected M_r . Alternatively, a different translation start site to that predicted may be used. Members of the protein phosphatase family are also known to be subject to alternative splicing to yield different isoforms (20–22), but whether this is a feature of IA-2-related phosphatases is not known. Clearly, amino acid sequence data on the precursor to 40,000- M_r fragments are required to define the precise relationship of this antigen to IA-2.

We have previously shown that antibodies in IDDM patients' sera to 37,000- M_r fragments are strongly correlated with those to 40,000- M_r fragments (15), suggesting that these are antigenically similar. However, data presented here identify antibodies in sera from nondiabetic relatives that can distinguish between the two fragments (Fig. 1 A). The precursors can also be separated by binding to immobilized lectins, indicating that these have different carbohydrate contents. Trypsinization of

IA-2ic or the complete IA-2 protein did not generate 37,000- M_r fragments. However, recombinant IA-2ic was able to block autoantibody binding to 37,000- M_r fragments, although with some sera blocking was incomplete. These observations indicate that the 37,000- M_r fragments are unlikely to be derived from IA-2, although the ability of IA-2ic to block autoantibody binding to the fragments does suggest that the antigen is structurally similar to another member of the protein tyrosine phosphatase family.

A number of potential islet cell antigens have been identified by screening of islet cDNA libraries with IDDM patients' sera, including carboxypeptidase H and ICA 69 (23, 24). However, to date, these have not corresponded to antigens detected by analysis of antibody reactivity with proteins in islet extracts, and the expressed recombinant proteins do not always show reactivity with patients' sera in immunoprecipitation reactions (25). Neither GAD nor insulin have been identified by the library screening approach. Diabetes-associated antibodies to these antigens are known to bind epitopes that are highly dependent on protein conformation (26, 27), and these may not be displayed by the library. If the 40,000- M_r fragments of islet antigen are indeed equivalent to IA-2 and ICA512, then this protein will represent a rare case of an islet antigen identified by both immunochemical and molecular biological approaches. We have shown in different populations that antibodies to the 37k and 40k antigens are very closely associated with diabetes development (3–6). The finding that the 40,000- M_r fragments of this antigen are related to the cloned proteins IA-2 and ICA512 will simplify assessment of the predictive value of these antibodies in larger populations. Furthermore, the availability of suitable amounts of recombinant antigen will allow studies to define the role of the protein in pathogenesis of disease and in its potential use in disease prevention therapies.

Acknowledgments

These studies were supported by grants from the British Diabetic Association, the Juvenile Diabetes Foundation International (194116), and the Royal Society. M. R. Christie is a Royal Society University Research Fellow.

References

- Harrison, L. C. 1992. Islet cell autoantigens in insulin-dependent diabetes: Pandora's box revisited. *Immunol. Today*. 13:348–352.
- Christie, M. R., J. A. Hollands, T. J. Brown, B. K. Michelsen, and T. L. Delovitch. 1993. Detection of pancreatic islet 64,000 M_r autoantigens in insulin-dependent diabetes distinct from glutamate decarboxylase. *J. Clin. Invest.* 92:240–248.
- Christie, M. R., R. Y. M. Tun, S. S. S. Lo, D. Cassidy, T. J. Brown, J. Hollands, M. Shattock, G. F. Bottazzo, and R. D. G. Leslie. 1992. Antibodies to glutamic acid decarboxylase and tryptic fragments of islet 64kD antigen as distinct markers for the development of insulin-dependent diabetes. Studies with identical twins. *Diabetes*. 41:782–787.
- Bingley, P. J., M. R. Christie, E. Bonifacio, R. Bonfanti, M. Shattock, M. Fonte, G. F. Bottazzo, and E. A. M. Gale. 1994. Combined analysis of autoantibodies improves prediction in islet cell antibody positive relatives. *Diabetes*. 43:1291–1296.
- Christie, M. R., S. Genovese, D. Cassidy, E. Bosi, T. J. Brown, E. A. M. Gale, E. Bonifacio, and G. F. Bottazzo. 1994. Antibodies to islet 37k-antigen, but not to glutamate decarboxylase, discriminate rapid progression to insulin-dependent diabetes mellitus in endocrine autoimmunity. *Diabetes*. 43:1254–1259.

- Genovese, S., P. J. Bingley, E. Bonifacio, M. R. Christie, M. Shattock, R. Bonfanti, R. Foxon, E. A. M. Gale, and G. F. Bottazzo. 1994. Combined analysis of IDDM-related autoantibodies in healthy schoolchildren. *Lancet*. 344:756.
- Lan, M. S., J. Lu, Y. Goto, and A. L. Notkins. 1994. Molecular cloning and identification of a receptor-type protein tyrosine phosphatase, IA-2, from human insulinoma. *DNA Cell Biol.* 13:505–514.
- Rabin, D. U., S. M. Pleasic, J. A. Shapiro, H. Yoo-Warren, J. Oles, J. M. Hicks, D. E. Goldstein, and P. M. M. Rae. 1994. Islet antigen 512 is a diabetes-specific islet autoantigen related to protein tyrosine phosphatases. *J. Immunol.* 152:3183–3188.
- Christie, M., M. Landin Olsson, G. Sundkvist, G. Dahlquist, Å. Lernmark, and S. Baekkeskov. 1988. Antibodies to a Mr-64000 islet cell protein in Swedish children with newly diagnosed type 1 (insulin-dependent) diabetes. *Diabetologia*. 31:597–602.
- Christie, M. R., D. Daneman, P. Champagne, and T. L. Delovitch. 1990. Persistence of antibodies to 64000-Mr islet cell protein after onset of Type 1 diabetes. *Diabetes*. 39:653–656.
- Christie, M. R., T. J. Brown, and D. Cassidy. 1992. Binding of antibodies in sera from type 1 (insulin-dependent) diabetic patients to glutamate decarboxylase. Evidence for antigenic and non-antigenic forms of the enzyme. *Diabetologia*. 35:380–384.
- Brundstedt, J., J. H. Nielsen, Å. Lernmark, and the Hagedorn Study Group. 1984. Isolation of islets from mice and rats. In *Methods in Diabetes Research. Laboratory Methods*, Vol. 1. C. J. Larner and S. Pohl, editors. John Wiley & Sons, Inc., New York. 245–258.
- Karlsen, A. E., W. Y. Fujimoto, P. Rabinovitch, S. Dube, and Å. Lernmark. 1991. Effects of sodium butyrate on proliferation-dependent insulin gene expression and insulin release in glucose-sensitive RIN-5AH cells. *J. Biol. Chem.* 266:7542–7548.
- Program Manual for the Wisconsin Package, Version 8. September 1994. Genetics Computer Group, Madison, WI.
- Christie, M. R., G. Vohra, P. Champagne, D. Daneman, and T. L. Delovitch. 1990. Distinct antibody specificities to a 64-kD islet cell antigen in type 1 diabetes as revealed by trypsin treatment. *J. Exp. Med.* 172:789–794.
- Rabin, D. U., S. M. Pleasic, R. Palmer-Crocker, and J. A. Shapiro. 1992. Cloning and expression of IDDM-specific human autoantigens. *Diabetes*. 41:183–186.
- Lu, J., A. L. Notkins, and M. S. Lan. 1994. Isolation, sequence and expression of a novel mouse brain cDNA, mIA-2, and its relatedness to members of the protein tyrosine phosphatase family. *Biochem. Biophys. Res. Commun.* 204:930–936.
- Baekkeskov, S., J. H. Nielsen, B. Marner, T. Bilde, J. Ludvigsson, and Å. Lernmark. 1982. Autoantibodies in newly diagnosed diabetic children immunoprecipitate pancreatic islet cell proteins. *Nature (Lond.)*. 298:167–169.
- Baekkeskov, S., G. Warnock, M. Christie, R. V. Rajotte, P. Mose-Larsen, and S. Fey. 1989. Revelation of specificity of 64K autoantibodies in IDDM sera by high resolution 2D gel electrophoresis. Unambiguous identification of 64K target antigen. *Diabetes*. 38:1133–1141.
- Walton, K. M., and J. E. Dixon. 1993. Protein tyrosine phosphatases. *Annu. Rev. Biochem.* 62:101–120.
- Pan, M.-G., K. P. Rim, T. Florio, and P. J. S. Stork. 1993. Cloning and expression of two structurally distinct receptor linked protein tyrosine phosphatases generated by RNA processing from a single gene. *J. Biol. Chem.* 268:19284–19291.
- Lefrançois, L., M. L. Thomas, M. J. Bevan, and I. S. Trowbridge. 1986. Different classes of T lymphocytes have different mRNAs for the leukocyte-common antigen, T200. *J. Exp. Med.* 163:1337–1342.
- Castano, L., E. Russo, L. Zhou, M. A. Lipes, and G. S. Eisenbarth. 1991. Identification and cloning of a granule autoantigen (carboxypeptidase-H) associated with type 1 diabetes. *J. Clin. Endocrinol. & Metab.* 73:1197–1201.
- Pietropaolo, M., L. Castañón, S. Babu, R. Buelow, Y.-L. S. Kuo, S. Martin, A. Martin, A. C. Powers, M. Prochazka, J. Naggert, et al. 1993. Islet cell autoantigen 69kD (ICA 69). Molecular cloning and characterization of a novel diabetes-associated autoantigen. *J. Clin. Invest.* 92:359–371.
- Lampasona, V., M. Ferrari, E. Bosi, M. R. Pastore, P. J. Bingley, and E. Bonifacio. 1994. Sera from patients with IDDM and healthy individuals have antibodies to ICA69 on western blots but do not immunoprecipitate liquid phase antigen. *J. Autoimmun.* 7:665–674.
- Kim, J., M. Namchuck, T. Bugawan, Q. Fu, M. Jaffe, Y. Shi, H.-J. Aanstoot, C. W. Turck, H. Erlich, V. Lennon, and S. Baekkeskov. 1994. Higher autoantibody levels and recognition of a linear NH₂-terminal epitope in the autoantigen GAD₆₅, distinguish stiff-man syndrome from insulin-dependent diabetes mellitus. *J. Exp. Med.* 180:595–606.
- Wilkin, T. J., I. Mirza, and M. Armitage. 1988. Insulin autoantibody polymorphism with greater discrimination for diabetes in humans. *Diabetologia*. 31:670–674.